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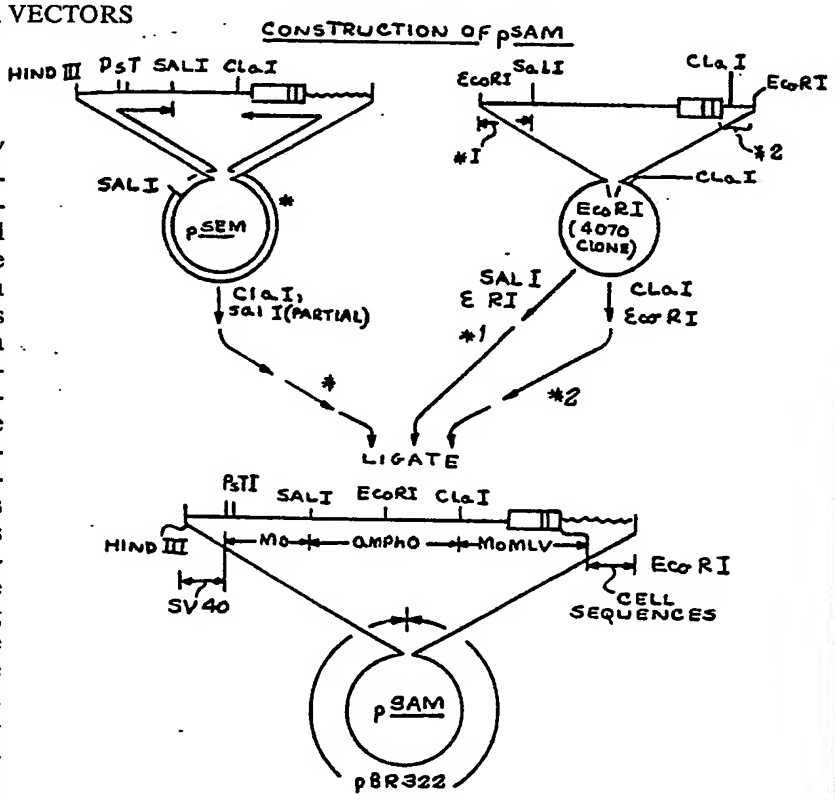
(57) Abstract

Recombinant DNA constructs include 5′ and 3′ retroviral LTR sequences, a genome packaging sequence, a promoter sequence and a eukaryotic cDNA sequence under the promotional control of the promoter sequence. By making the retroviral sequences of the construct deficient in coat protein-encoding sequences, the construct is by itself incapable of packaging its genome as a retrovirion. The promoter sequence may be a viral LTR sequence. Alternatively the promoter sequence may be a nonviral promoter linked to the cDNA sequence, promoting the cDNA irrespective of cellular mechanisms which affect viral sequence expression. The retroviral genomes corresponding to the constructs are rescued as complete packaged virions by helper virus vectors which encode the coat proteins that are needed to package the genomes corresponding to the constructs. Helper virus vectors that are deficient in packaging sequences do not package their own genomes, and cells incorporating such a deficient helper virus vector and also a construct shed only retrovirions that are infectious a single time. Helper viral vectors which produce amphotropic proteins package recombinant retrovirions that are infective of cells of a variety of species. Cells infected with recombinant retrovirions are transplantable into an animal to comprise a portion of the somatic cells of the animal and express recombinant gene product therein. Recombinant retrovirionsaccording to the invention, if inoculated into the peripheral fluid of a host animal, will infect and genetically alter the somatic cells of the animal.



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RETROVIRAL GENE TRANSFER VECTORS

The present invention relates generally to the use of retroviral vectors for transferring genetic information into cells.

BACKGROUND OF THE INVENTION

The ability to introduce eukaryotic genes into eukaryotic cells has contributed much to our current knowledge of the mechanism and regulation of gene expression. A variety of methods have been used to transfer foreign genetic material into cells, each method having certain advantages and limitations.

The widely used calcium phosphate-mediated gene-transfer (transfection) technique is relatively inefficient and is limited to use in cultured cells. Introduction of foreign sequences by microinjection into target cells is efficient but requires sophisticated instrumentation.

An alternative approach for transferring genes into cells involves the use of viral vectors. The commonly used papovavirus vectors, such as simian virus 40 (SV40), however, have major limitations: (i) the length of the foreign DNA segment cannot exceed 2.5 kilobase pairs (kbp) and (ii) papovaviruses have a limited host range.

Because of their unique structure and mode of propagation, retroviruses (a class of viruses having a single-stranded RNA genome and a particular life cycle) appear to be ideally suited as gene-transfer vehicles. Some of their unique properties include (i) the RNA viral genome is efficiently transmitted to the recipient cells and integrated into the chromosome(s) as double-stranded DNA, (ii) integration is specific with respect to the viral genome, (iii) the plasticity of the viral genome permits packaging of DNA inserts of up to at least about 7 kbp, (iv) retroviruses have a wide host range and can infect a variety of cell types, and (v) viral long terminal repeats (LTRs) provide efficient

signals for initiation and termination of transcription. Several groups have recently used retroviral DNA vectors to generate infectious virus containing either the herpes simplex virus thymidine kinase (TK) gene or the Escherichia coli guanine phosphoribosyltransferase gene (Eco gpt).

It is of particular interest to use retroviruses for imparting genetic characteristics to vertebrate cells, including human cells for a variety of purposes. A genetic disease might be corrected, for example, by introducing genetic material into a sufficient population of somatic cells of a living animal, including a human. A valuable gene-encoded product, such as a pharmaceutically useful peptide, might be farmed in a host animal. Animals that are raised as a source of food might be genetically altered so as to be more efficient protein producers. Desirable characteristics of a retrovirus that is to be used for introducing characteristics into eukaryotic cells include flexibility that allows the retrovirus to be used for introducing genetic characteristics into a wide variety of cell types from a variety of species, the inability of the retrovirus to cause a spreading infection or to otherwise bring about deleterious changes in the infected cell or in the animal as a whole, efficiency of gene transfer into cells, and efficient encoding of the desired genetic characteristic carried by the retrovirus.

SUMMARY OF THE INVENTION

Novel retroviral vectors are constructed and used to carry genetic information to the cells through infection. DNA constructs are initially assembled from proviral DNA sequences (DNA sequences corresponding to RNA sequences of the retroviral genome) as well as DNA sequences obtained from nonviral sources, including genes encoding proteins that, when expressed, impart novel characteristics to the cells. The viral sequences

used in the constructs include long term repeating (LTR) sequences which include sequences necessary for the genome to insert itself in DNA form (proviral form) into a cell's genome and also provide efficient signals for initiation and termination of transcription. The constructs also retain viral sequences that direct packaging of viral genome RNA. In order to avoid producing recombinant virions capable of generating a spreading infection, in preferred embodiments of the invention, regions encoding viral proteins are removed and/or truncated.

The DNA constructs are introduced into cells by conventional methods, such as calcium phosphate transfection, and a gene product is expressed in the cells. The constructs, if deficient in packaging protein-encoding sequences, can be "rescued" as packaged retrovirions from the cells by introducing into a construct-transfected cell a "helper" retroviral vector. These helper viral vectors express packaging proteins within the cells that are necessary for viral replication. The helper virus vector is rendered defective by removing its own packaging sequences, so that it produces the needed proteins to package the construct-encoded RNA genome but does not package its own genome.

It is also preferred that the helper retroviral vector be rendered defective so that it cannot package itself into the gene of the host cell. The rescued retrovirion that is obtained from the construct is efficiently infectious into eukaryotic cells, and the packaged virus inserts its genetic information linearly into the DNA of the host cell. However, lacking necessary protein-encoding sequences, it is incapable of repackaging itself and thereby induce a spreading infection. The recombinant retrovirus constructs can be used to carry nucleic acid chains of substantial length, e.g., up to about 7 kilobase pairs (kbp), into cells, and both selectable genes and nonselectable genes may be

carried into cells. If the RNA genome corresponding to the construct is packaged with an amphotropic helper virus, the resulting recombinant retrovirion exhibits infectivity that crosses biological family and even phylum lines.

The recombinant retrovirions according to the invention may be used to infect vertebrate animal cells in vitro, and the infected cells may then be transplanted into an animal where the infected cells express the protein product that is encoded by the genetic material that was carried to the cells by the retrovirion.

Genetic material may be inserted into retroviral vectors under the control of the LTR viral sequences. Alternatively, it is found that genes may be inserted into retroviral vectors under control of their own promoter sequences, in which case, they are expressed in cells irrespective of the degree of functionality of the viral sequences themselves within the cells.

IN THE DRAWINGS

FIGURE 1 is a schematic representation of the assembly of DNA constructs, pLPAL and pLPL, which embody various features of the present invention;

FIGURE 2 is a schematic representation of the assembly of a helper virus construct, designated pSEM, embodying various features of the present invention;

FIGURE 3 is a schematic representation of an amphotropic helper virus construct, designated pSAM, embodying various features of the present invention;

FIGURE 4a is a schematic representation of construction of a rat growth hormone mini-gene;

FIGURE 4b is a map of the rat growth hormone mini-gene;

FIGURE 5a is a schematic representation showing insertion into the pLPL construct of FIG. 1 of the rat growth hormone mini-gene of FIGURE 4b; the mini-gene

being inserted in a manner such that expression of the rat growth hormone gene is under the control of the endogenous rat growth hormone gene promoter; and

FIGURE 5b is a map of the viral genome-encoding portion of the construct of FIGURE 5a.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

DEFINITIONS

The following terms are defined below as they are used in this application:

Retrovirus -- a virus having a single-stranded RNA genome and having a life cycle described in Kornberg, DNA Replication, W.H. Freeman & Co., San Francisco (1980) pp. 596-598.

Provirus -- the double-stranded counterpart of the retroviral genome that comprises an essential stage of the life cycle of a retrovirus.

Virion -- a packaged, infectious viral genome, including retroviral genomes that are packaged with proteins that are not encoded by their own nucleic acid sequences.

Construct -- a recombinant DNA assemblage including viral sequences.

Retroviral vector -- a vehicle for introducing genetic material into a cell that includes retroviral sequences; the term is used herein to generally include DNA and RNA material and in packaged or non-packaged form.

Helper virus -- a virion capable of infecting a cell and encoding replication protein and packaging genomic RNA for further propagation when introduced into a cell.

Helper virus vector -- a helper virus as defined above, and including plasmid DNA containing the viral gene which can be transfected into cells but unable to propagate.

Production of gene-carrying, infectious, recombinant retroviral vectors according to the present

invention begins with the assembly of a DNA construct that contains DNA sequences corresponding to RNA sequences of a parent(s) retrovirus as well as additional DNA sequences, usually including a DNA sequence that encodes a desired protein product. The DNA construct generally includes LTR viral sequences of the parent retrovirus including 5' and 3' sequences that are necessary for insertion of the genome in its proviral form into the chromosomal DNA of the host cell. Portions of the LTRs that signal initiation and termination of transcription of protein encoding segments may also be included in the construct, depending upon its purpose. Importantly, the construct includes sequences necessary for assemblying proteins to package its corresponding RNA genome. The DNA construct preferably excludes at least some of the viral sequences that encode viral proteins, making it impossible for the viral RNA genome to be packaged as an infectious virion without the assistance of a helper viral vector. This helps to assure that any retrovirion that is eventually packaged with the assistance of a helper viral vector will be capable of only an initial infection and will be incapable by itself of inducing a spreading infection.

Furthermore, removal of protein-encoding segments very substantially reduces the total length of retroviral sequences in the construct, allowing room between the 5' and 3' LTR sequences for long foreign sequences to be included in the construct while keeping the recombinant retroviral sequences within the construct to a length compatible with RNA genome packaging. Retroviral vectors according to the present invention may be packaged up to a length of about 9 kbp, and by retaining only about 2-3 kbp of the viral sequences, the construct can carry up to 6-7 kbp of foreign genetic material within the region bounded by the 5' and 3' insertion sequences. The extended length of the foreign sequence that can be inserted represents

an important advantage of retroviral vectors for gene transfer applications.

The invention further provides recombinant retroviral constructs from which all protein-encoding sequences are deleted or truncated in a manner that assures that no coat protein or coat protein fragments are expressed within a cell that incorporates the viral vector sequences. Complete impairment of the ability to encode coat proteins eliminates the continuing antigenicity of the packaged retrovirion derived from the construct. This is important when the retrovirion is used to directly infect an animal, as hereinafter described. If initial infection by a recombinant retrovirion does not induce antibody production, subsequent infections by additional recombinant retrovirion is permitted.

The various DNA fragments used to assemble the DNA construct are cut and ligated by procedures that are conventional in the art and will not be described in detail herein. Proviral DNA is obtained, for example, according to the method of Verma, I.M. et al., Proc. Natl. Acad. Sci USA 77, 1773-1777 (1980) and Van Bevern et al. Cell 27, 97-108 (1981).

The double-standard DNA construct generally contains plasmid sequences because at each step of the construction, multiple copies of the intermediate recombinant DNA constructs are obtained by raising them in plasmid form in a prokaryotic cell, such as E. Coli. The amplifications of intermediate constructs also represents purifications of the intermediate constructs. The plasmid sequences that are outside of the bracketed viral LTR sequences do not become part of the retroviral genome and need not be considered for genome packaging considerations; e.g., when considering the length of an inserted segment.

A DNA segment that encodes a gene product is selected according to the intended end utility of the

retroviral vector. In some cases, the proteinaceous product that is encoded by the selected DNA segment may impart a selectable characteristic to the cell. For example, described herein as an example of the present invention is the introduction into cells of the human hypoxanthine phophoribosyltransferase (hHPRT) gene by retroviral infection. Genetic HPRT deficiency in humans is associated with the devastating Lesch-Nyhan syndrome and forms of gouty arthritis. An infectious retroviral vector incorporating the hHPRT gene for the purpose of incorporating the gene into cells may be used to ameliorate the genetic deficiency. The HPRT gene further imparts a selectable marker to the infected cells, facilitating selection of cells that have incorporated the gene.

If the gene of interest is not readily selectable, e.g., growth hormone (GH), it is generally preferred that the retroviral vector include, in addition to the gene of interest, a selectable marker for purposes of amplification and identification.

The DNA constructs are transfectable into eukaryotic cells by standard technique, including the well known calcium phosphate precipitation procedure, Graham, F.L. et al., Virology 52, 456-467 (1973). A cell that is transfected with the construct may express the products of the genetic sequences carried by the construct. However, at this stage, gene product expression is primarily important as a vehicle for selecting cells that are transfected because many of the advantages that accrue through the use of retroviral vectors are not fully realized at this stage. Thus, the transfected cell is generally considered only an intermediate step in the method of the present invention.

Recombinant retrovirions, from constructs that are purposefully assembled so that they are not self-packaging whereby the retrovirions may be used for one-time infection of other cells, are obtained

("rescued") from the construct-transfected cells with proteins expressed by "helper virus" vectors that are also introduced into the transfected cells. The helper viral vector encodes proteins that are not encoded by the construct but which are necessary for packaging the recombinant RNA viral genome corresponding to the DNA construct. The helper viral vector is also of retroviral derivation and is related to the parent retrovirus from which the DNA construct was assembled, at least to the extent that its packaging proteins will package the recombinant retroviral genome.

As defined above, "retroviral vector" herein is used to encompass several forms of retroviral genetic material, and helper vector rescue may be accomplished in a variety of manners, depending upon the requirements of the particular application. A cell line may be pre-altered, either by infection or transfection, so as to incorporate a helper virus vector as DNA within its genome, and thereby expressing helper viral coat proteins. When a construct is subsequently transfected into such a pre-altered cell line, the helper virus vector-expressed coat proteins are used to package the RNA genome of the construct. Alternatively, a construct that contains genetic material of interest may be transfected into a cell line and the cell line subsequently infected with a helper retrovirion. As a further alternative, helper viral constructs and recombinant retroviral constructs may be cotransfected into a cell line. In all cases, packaged recombinant retrovirion is shed from cells that incorporate both the construct and the helper virus vector.

The helper viral vector that is used in virion rescue is preferably selected or constructed so as to be innocuous in the end use of the recombinant infectious retrovirion. For example, if the recombinant retrovirion is to be used to carry genetic information into somatic cells of a living animal, a helper virus

may be selected which is known to cause no adverse effects in the animal. Alternatively, and in many instances preferably, the helper virus vector is itself defective, lacking sequences needed to package its own genome as an infectious virus. The helper viral vectors are made defective by deleting their packaging sequences while retaining their coat protein-encoding segments. Cells transfected with a recombinant construct and also incorporating a defective helper viral vector will shed only recombinant retrovirions having genomes corresponding to the construct and that contain the desired genetic material. These retrovirions, though directly infectable into cells for a one-time infection, will not subsequently repackage their genome, and thus, cannot by themselves create a spreading infection.

The DNA constructs produced according to the methods of the invention are further manipulatable to incorporate additional genetic information. For example, retroviruses which are initially constructed so as to carry a gene that encodes a selectable protein may be further altered to incorporate a gene that encodes a nonselectable protein. This permits cells to which the desired nonselectable gene has been imparted by the retroviral vector to be selected through growth in an appropriate medium from cells which have not incorporated the genetic information carried by the vector.

Retroviral LTRs include strong promoter sequences, and in many instances it is desirable to insert a product-encoding DNA sequence into the construct under the promotional control of the LTR promoter sequences. However, it may be the case that although a retroviral vector will incorporate its genetic information into the genome of an infected cell, the incorporated genetic information will not be expressed to any significant extent due to various cellular control mechanisms. In accordance with an

important aspect of the present invention, it is found that product-encoding DNA segments may be incorporated into retroviral constructs linked to and under the control of their own promoter or of another nonviral promoter. A eukaryotic gene that is carried by a retroviral vector into a eukaryotic cell under the control of a nonviral promoter is found to express the gene products irrespective of the functionality of the viral genetic information incorporated into the cell. Self-promoted genes function independently of viral regulatory elements, such as transcription promoters, splice sites or polyadenylation signal. Thus, factors which reduce or totally suppress viral gene expression, which may be due to the failure of viral promoters or other regulatory sequences to be activated in particular cells, do not affect the expression of the gene promoted by its own transcriptional regulatory signals. Evidence that a retroviral vector can carry a protein-encoding segment into a cell under control of its own promoter is evidenced by the fact that encoding segments linked to their own promoter express product irrespective of their orientation (parallel or reversed) relative to the viral LTR promoter sequences.

Often, eukaryotic genes contain introns, i.e., noncoding segments. Because correct transcription of an intron-containing gene requires regulatory mechanisms present in the cell to which the gene is endogenous and which may be absent in the host cell, it is generally desirable to remove the introns prior to insertion of the gene into a retroviral vector so that the gene will be correctly expressed in whatever cell line the gene is carried by the retroviral vector. Removal of introns may be accomplished by production of cDNA by reverse transcription from messenger RNA. cDNA contains only protein-encoding sequences and therefore lacks upstream promoter sequences. In those cases where it is desirable to insert a eukaryotic gene into a retroviral

vector under the promotional control of a nonviral promoter, it is necessary to link a promoter to the cDNA in appropriate fashion.

The promoter sequence may be the natural promoter of the gene or may be another promoter selected for particular properties. For example, a promoter that is known to respond to external stimuli may be linked to a cDNA segment, permitting expression of the retroviral vector-inserted gene to be externally regulated after it is incorporated into the genome of a cell. Examples of externally regulatable promoter sequences include, but are not limited to, promoter sequences for the following genes: chicken transferrin gene, mouse metallothionein I gene, mouse metallothionein II gene, mammalian transferrin genes, mammalian histocompatibility genes, mammalian ceruloplasmin genes, mammalian glycolytic enzyme genes, mammalian albumin genes, mammalian immunoglobulin genes, mammalian interferon genes, and mammalian heat shock genes.

A further advancement of the present invention is the novel use of amphotropic, replication-competent helper viruses to rescue infectious retrovirions having the construct genome. If rescued with amphotropic helper viruses, the rescued virions are packaged in proteins, particularly envelope (env) proteins, that make them widely infective of cells of various species. By amphotropic is meant the ability to infect cells of relatively unrelated species, and herein the term "amphotropic" shall be used to refer to retroviral vectors which are infective across biological family lines. Certain retroviral vectors are even sufficiently amphotropic to cross phylum lines, e.g., between mammals and birds. Amphotropism in a retroviral vector gives the vector much wider utility, e.g., for imparting genetic characteristics, such as rapid growth, to a wide variety of domestic animals. It also allows a vector that is intended for gene therapy in humans to be

initially developed and grown in cell lines derived from a lower animal.

Among the important advantages afforded by retroviral vectors is the efficiency with which the genetic information of the retroviruses is incorporated into the genome of the cell. In certain retroviral infection protocols, substantially every cell is found to incorporate the retroviral genetic information. Another important advantage afforded by retroviral vectors is the general tendency to incorporate a single copy of the genetic material into the genome of each cell. If the cell to be infected is either a part of a living animal or is to be transplanted into a living animal, it is generally preferred that the cell contain a single copy of the genetic material.

An important use of retroviral vectors is for gene therapy in vertebrate animals, including fishes, birds and mammals. Retroviral vectors are expected to be useful in correcting genetic deficiencies in humans. As one method of performing gene therapy on an animal, a cell line is infected with recombinant retrovirion, and infected cells that are found to express the product(s) of a gene(s) carried into the cells by the retrovirion are transplanted into the animal. It is demonstrated that such genetically altered cells continue to express gene product within the recipient animal. Preferably, the cell line that is selected for use in this type of gene therapy is rapidly proliferating, e.g., the systemic stem cells of the bone marrow.

The invention will now be described in greater detail by way of specific examples.

EXAMPLE 1

Assembly of two constructs containing retroviral sequences and an hHPRT-encoding sequence is diagrammatically outlined in FIGURE 1. The recombinant plasmids were constructed using standard techniques as described in Maniatis, T. et al., Molecular Cloning: A

Laboratory Manual (Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.) and Verma et al. (1980) supra. In Figure 1, abbreviations of common restriction enzymes are P, Pst I; B, Bam HI; H, Hind III; E, Eco RI; R. Rsa I; Hp, Hpa I; S. Sal I. To reduce unwanted ligation products, the indicated fragments were treated with bovine alkaline phosphatase (BAP). Cross-hatched segments represent hHPRT cDNA, boxes with interior arrows represent viral LTRs with orientation indicated by the arrow (5' -- 3'), thin lines indicate viral sequences, thick lines indicate plasmid pBR322 sequences, and wavy lines indicate mouse cell sequences surrounding proviral DNA.

The cDNA clone of the gene for hHPRT was obtained from the SV40-based expression vector p4aA8 in which it was initially isolated, Jolly, D.J., et al., Proc. Natl. Acad. Sci. USA 80, 477-481 (1983). Viral LTRs were linked to the hHPRT cDNA such that the coding regions for viral proteins were either completely removed (gag and pol) or truncated (env); thus, no viral proteins would be expressed from the contructs. The 5' LTR and adjacent sequences were derived from a cloned Mo-MSV provirus, $pMSV_i$ IL, Van Beveren et al., Cell 27, 97-108 (1981), and the 3' LTR and adjacent sequences were obtained from cloned unintegrated Mo-MuLV DNA, pMLV-1, Berns et al., J. Virol; 36 254-263, (1980); Shinnick et al., Nature (London), 293, 543-548 (1981). Retained in the constructs are 5' sequences presumably involved in packaging of viral RNA, Shank, P.R. J. Virol 36, 450-456 (1980). The hHPRT gene is situated so that the ATG start codon is in approximately the same position as the start codon for the gag polyprotein in the parental virus.

Two potential polyadenylylation (poly A) signals, which are expected to terminate transcription and thereby reduce the efficiency of the construct, exist near the 3' end of the Pst I to Bam HI hHPRT cDNA

fragment. Thus, in addition to the pLPAL construct containing these sites, another construct, pLPL, was made in which these sites were removed and replaced with Mo-MuLV sequences. In pLPL, the hHPRT cDNA was joined to the viral env gene downstream of the env splice acceptor junction and start codon. Thus, pLPL should be transcribed as a single RNA species of about 4.0 kb, which would encode hHPRT.

Cell lines and tissue cultures used in this Example are as follows. The 208F hHPRT$^-$ clonal cell line was derived from Fischer rat cells by selection in 6-thioguanine, Quade, K., Virology 98, 461-465 (1979). The BALB 3T3 hHPRT$^-$ cell line 2TGOR, Jha, K.K. et al., Somat. Cell Genet. 6, 603-614 (1980), was a gift of H. L. Ozer (Hunter College, New York). The hHPRT$^+$ rat cell line WA, derived from Wag-Rij rats, was a gift from M. Maszynsky (Case Western University) and G. Rosenfeld (University of California at San Diego). All cell lines, including NIH 3T3 TK$^-$ cells, Wei, C. et al., J. Virol. 39, 935-944 (1981); SV40-transformed hHPRT$^-$ Lesch-Nyhan (LNSV) cells, Bakay, B. et al., J. Cell Sci. 17, 567-578 (1975); GM637 SV40-transformed normal (hHPRT$^+$) human fibroblasts, Tan, Y. H. et al. J. Gen. Virol. 34, 401-411 (1977); and D98 hHPRT$^-$ HeLa cells, Francke, U., Chromosoma 41, 111-121 (1973) were maintained in Dulbecco-Vogt modified Eagle's medium, 10% fetal bovine serum (Sterile Systems, Logan, UT). hHPRT$^+$ cells were selected in the same medium containing 30 μM hypoxanthine/1.0 μM amethopterin/20 μM thymidine (HAT medium).

For transfecting cells with the constructs, hHPRT$^-$ cells were plated at 5 X 10^5 cells per 5-cm dish on day one. On day two, 0.5 μg of uncut recombinant plasmid DNA and 8 μg carrier DNA (from NIH 3T3 TK$^-$ cells) were transfected into the cells using the calcium phosphate precipitation procedure of Graham, F.L. Virology 52, 456-467 (1973); Corsaro, C.M. et al.,

Somat-Cell Genet. 7, 603-616 (1981). On day three, the cells were split 1:5 into HAT medium, and every three days thereafter, the medium was replaced with fresh HAT medium. For quantitation, colonies were stained on day nine with Coomassie blue (1 g/liter in 50% methanol/10% glacial acetic acid/40% water). In a transfection assay, recombinant plasmids, pLPA, pLPAL, and pLPL, conferred HAT resistance (HAT^r) to HAT-sensitive (HAT^s) $hHPRT^-$ 208F rat cells. The efficiency of HAT^r colony production was the same for the constructs as for the original SV40-hHPRT hybrid plasmid p4aA8 in which hHPRT is expressed from a SV40 promoter (400-700 colonies per $\mu g \cdot 10^6$ cells). No HAT^r colonies were observed in control 208F cells.

Cells transfected with the hHPRT-containing viral constructs are unable to produce virus because the coding regions for the viral proteins that are necessary for viral replication have been deleted. However, defective viral genomes were rescued with replication-competent helper virus. hHPRT-expressing virus was rescued from cells using replication-competent helper virus, either Moloney murine leukemia virus (Mo-MuLV), Fan, H. et al., J. Virol., 14 421-429 (1974), FBJ murine leukemia virus (FBJ-MuLV), Curran, T. et al., Virology 116, 221-235 (1982), or 1504A amphotropic virus Rasheed, S. et al., J. Virol. 19, 13-18 (1976). The later amphotropic virus is widely infective in a variety of vertebrate cells, having an envelope protein that enables it to infect, for example, chicken, rodent as well as human cells.

Medium from helper virus-producing cells was filtered through a 0.45 μm filter and applied to cells at a multiplicity of about 1 in the presence of Polybrene (Sigma) at 4 μg/ml. The cells were passaged 3 days after infection, medium was replaced with fresh medium on day five, and supernatant was harvested on day six from nearly confluent cells. The supernatant was

centrifuged at 5,000 x g for 7 min. to remove cells, and aliquots of the supernatant were applied to 208F $hHPRT^-$ and NIH 3T3 TK^- cells (plated the day before at 5 x 10^5 per 5-cm dish) in medium containing Polybrene at 4 μg/ml. $hHPRT^+$ 208F cells were scored as in the transfection assay, and Mo-MuLV and FBJ-MuLV were quantitated by using the XC plaque assay of Rowe, W.P. et al., Virology 42, 1136-1139, on NIH 3T3 TK^- cells. 1504A amphotropic virus is XC-plaque negative and was not quantitated.

In initial experiments, $hHPRT^-$ rat cells transfected with pLPAL or pLPL were infected with helper virus and the virion produced by these cells was able to confer HAT resistance to $hHPRT^-$ cells. Furthermore, transmissible hHPRT-virus was also obtained when molecularly cloned helper virus DNA was cotransfected with pLPAL or pLPL. Under both of these conditions, the relative titer of hHPRT-expressing virus was uniformly low (1% of helper virus titer). Analysis of the transfected cells revealed a number of aberrant forms of viral RNA, which may not be properly packaged or may produce noninfectious virions.

In an attempt to obtain high hHPRT-expressing virus titers, the following strategy was used. $hHPRT^-$ rat cells were infected with hHPRT-expressing virion obtained from helper virus-infected pLPAL- or pLPL-transfected rat cells. HAT^r clones that did not produce helper virus (nonproducer cells) were isolated. Nonproducer cell lines were superinfected with helper virus and the culture medium was assayed for

hHPRT-expressing virus and helper virus. As shown in Table 1 below:

TABLE 1

Rescue of HPRT-expressing virus*

Cells infected	Mo-MuLV cfu/ml	Mo-MuLV pfu/ml	FBJ-MuLV cfu/ml	FBJ-MuLV pfu/ml	1504A cfu/ml	1504A pfu/ml
208F	<10	$10^{4.4}$	<10	$10^{3.8}$	<10	<10
208F-LPAL	$10^{1.7}$	$10^{4.6}$	$10^{2.0}$	$10^{3.9}$	$10^{1.7}$	<10
208F-LPL	$10^{3.3}$	$10^{4.8}$	$10^{3.0}$	$10^{4.1}$	$10^{3.5}$	<10
2TGOR-LPL	$10^{5.3}$	$10^{6.3}$				

*hHPRT-expressing virus was rescued from cells by using various helper viruses. Results are expressed as $hHPRT^+$ colony-forming units (cfu) and helper virus XC plaque-forming units (pfu). The amphotropic virus 1504A does not form plaques in the XC assay and was not quantitated.

Mo-MuLV, FBJ-MuLV, and the amphotropic virus were able to rescue hHPRT-expressing virus. Although hHPRT-expressing virus could be rescued from cells infected with either LPAL or LPL, the titer from cells infected with the LPAL virion that contains the hHPRT polyadenylylation signals was no more than 10% of that cells infected with LPL from which these signals had been deleted. This result confirms the hypothesized advantage of removing poly A sites from retroviral constructs. Averaged over many experiments, the hHPRT-expressing virus/helper virus ratio using the LPL-infected nonproducer line was about 10%.

The relatively low titers of hHPRT-expressing virus rescued from 208F rat cells refects the inefficient replication of murine retroviruses in rat cells. In another experiment, a $hHPRT^-$ murine cell line, 2TGOR, was infected with virion from rat 208F cells producing hHPRT-expressing virion (LPL) and Mo-MuLV. HAT^r cells were selected and passaged once to allow for helper virus spread. Supernatant from a

nearly confluent dish of these hHPRT$^+$ mouse cells had a titer of 2 x 10^5 hHPRT-expressing virus and 2 x 10^6 of the Mo-MuLV helper virus (Table 1). Thus, high titers of hHPRT-expressing virus can be generated in an appropriate cell line.

One important aspect of the present invention is the selection in certain applications of an amphotropic helper virus that rescues recombinant viruses in a manner that the rescued virions are generally infective in vertebrate cells, and particularly those that are infective of human cells. If a viral genome is packaged with proteins encoded by an amphotropic helper virus vector, either natural or recombinant, even though the retrovirus from which the recombinant genome is derived is specifically infectious of a single species or limited number of related species, the packaged recombinant retrovirion is able to infect a variety of cell types from a variety of species. This is because it is the packaging proteins, rather than the RNA genome, that determines which cells the recombinant virus is able to infect. Accordingly, one of the helper virions selected for these experiments was the above-mentioned amphotropic helper virus 1504A which is able to infect human cells.

All hHPRT$^-$ human cells tested, including D98 HeLa cells, SV40-transformed Lesch Nyhan (LNSV) fibroblasts, and Epstein-Barr virus-transformed Lesch-Nyhan lymphoblasts, could be converted from HAT^s to HAT^r by infection with culture medium from LPL-infected nonproducer rat cells that were superinfected with amphotropic virus. The efficiency of formation of HAT^r colonies was similar to that obtained for infection of 208F rat cells.

The hHPRT activity of the hHPRT-expressing virus-infected LNSV cells was analyzed to ascertain whether authentic human hHPRT protein was present and at what levels. Several rat cell lines that had been

transfected or infected with the hHPRT-expressing viral vectors were also analyzed. Both the LNSV cells and rat 208F cells infected with hHPRT-expressing virus express hHPRT protein that comigrates on polyacrylamide gels with authentic human hHPRT from HeLa or GM367 cells and migrates differently from rat hHPRT from WA cells. Control LNSV cells and 208F $hHPRT^-$ rat cells have undetectable activity in this assay. The levels of enzyme activity in LNSV and rat cells infected with hHPRT-expressing virus obtained from the LPL construct are similar to those of control $hHPRT^+$ human cells. 208F rat cells infected with hHPRT-expressing virus from the LPAL construct display human hHPRT activity but at a significantly lower level than the LPL-infected cells.

The RNA from cells either transfected or infected with the hHPRT retroviral vectors was analyzed to determine whether the RNA patterns corresponded to those predicted from the DNA organization of the vectors. Two probes were used, one to hHPRT cDNA and the other to the Mo-MSV LTR. The LTR probe does not hybridize to RNA transcripts from control 208F cells while the hHPRT probe hybridizes slightly to control cell RNA. A complex pattern of RNA transcripts hybridizing to either the LTR or hHPRT probe was observed in cells transfected with the vectors. RNA blot analysis of RNA from cells infected with hHPRT virus, on the other hand, produced a readily interpretable pattern because viral integration is precise with respect to the viral genome. In cells infected with hHPRT-expressing virus from pLPAL-LPAL-transfected cells, two species of RNA hybridizing to both probes were identified. The size of the larger RNA species corresponds to the full-length RNA transcript expected from LPAL (3.9 kb). The smaller species of RNA (1.6-1.9 kb) probably represents transcripts that initiated at the 5' LTR but terminated at the putative polyadenylylation signals in the hHPRT

cDNA fragment. In hHPRT(LPL)-expressing, virus-infected cells, only a single species of RNA hybridized to either the hHPRT probe or the LTR probe. The size of this RNA corresponds to the full length RNA (4.0 kb) expected from LPL if the normal retroviral transcriptional signals are used. The fact that all major species of RNA in hHPRT-expressing virus-infected cells hybridize to both viral LTR and hHPRT probes is consistent with the idea that transcripts initiate in the LTR and transcribe through the hHPRT cDNA sequences.

To determine the organization of chimeric proviral DNA in hHPRT-expressing virus-infected cells, the DNA in recipient LNSV cells was analyzed by using restriction endonucleases. The restriction endonuclease Sst I cleaves only once in each LTR of pLPL and does not cut in the SV40-based expression vector p4aA8. When high molecular weight DNA from LNSV cells was cleaved with Sst I and then hybridized to a hHPRT probe, two fragments of about 14.5 and 7.2 kbp were identified. These fragments presumably represent the endogenous hHPRT gene of LNSV cells. In cells transfected with the hHPRT expression vector p4aA8, an additional fragment larger than 25 kbp was detected, which is consistent with integration of the p4aA8 plasmid in a tandemly repeated unit, Perucho et al., Cell 22, 309-317 (1980), which would not have internal Sst I sites. Sst I-cleaved DNA from LNSV cells that were either transfected or infected with LPL vectors shows the control cell DNA fragments plus a single new fragment of about 3.9 kbp, as expected. Thus, like other retroviruses, the hHPRT-proviral DNA appears to have an uninterrupted 5' LTR-hHPRT-LTR 3' organization.

The size of the hHPRT-expressing virus is small, less then 4.5 kb, including both LTRs. It should be noted that only the construct segments from the 5' LTR through 3' LTR comprises the viral genome, the surrounding plasmid sequences that are used for

construct replication and selection comprising no portion of the genome that is transcribed from the construct and subsequently packaged. At the 5' end of the virus, 1.8 kb includes the 5' LTR, the tRNA binding site, the presumed packaging signal, and the hHPRT cDNA. About 0.7 kb at the 3' end includes the 3' LTR and sequences involved in the synthesis of plus-strand DNA during viral infection. Thus, only 2.5 kb of the viral sequences are required for hHPRT expression and virus rescue. Therefore, 6-7 kb of additional sequences could be added to the hHPRT-encoding virus without exceeding the limit for retroviral genome size. Hence, this vector can be used as a vehicle for other genes and carries a convenient and powerful selectable marker.

EXAMPLE 2

The helper viruses used in Example 1 are themselves infectious, being able to package their own genome and be shed as packaged viruses from infected cells. For certain applications, particularly for eventual end use for gene therapy in humans, it is undesirable to have infectious helper viruses which may have the potential to induce tumors. It may be noted, however, that many replication-competent viruses, like FBJ MULV virus, T. Curran, N. Teich, Virology 116, 221-235 (1982), have never been shown to cause any disease. Nevertheless, to avoid any likelihood of a helper virus inducing a tumor or other pathogenic condition, the invention provides defective helper viruses which cannot package their genomes but which encode proteins needed to package the recombinant retroviral vector. Construction of a defective helper virus pSEM is diagrammed in FIG 2. pMLV-intermediate is described by Berns et al., J. Virol. 36, pp. 254-263 (1980). pMLV-48 (MO-MLV provirus) is described by Bacheler et al., J. Virol. 37, p. 181-190 (1981)

Briefly, molecularly cloned MO-MLV DNA containing the coding region for gag-pol-env and 3'LTR

sequences are linked to a DNA fragment containing SV40 promoter and enhancer elements as well as splice donor-acceptor sites for viral coat protein. Thus the 5' LTR insertion and promoter sequences as well as the closely adjacent viral packaging sequences are replaced by SV40 insertion and promoter sequences with no adjacent packaging sequences. Replacement of the 5' LTR insertion segments with non retroviral sequences having promoter sequences eliminates the retroviral recombinations that frequently occur when both 5' and 3' LTR segments are present.

Rescue of a recombinant construct, such as pLPL, with a defective helper vector, e.g., by cotransfection, generates infectious LPL virion without generating any helper virus upon subsequent infection of recipient cells. A variety of sensitive assays designed to determine infectious helper virus particles gave negative results. It is estimated that titers of 10^4 LPL virus are released from cells cotransfected with pSEM. The titers can be increased if appropriate indicator cells are used for hHPRT assays.

EXAMPLE 3

The defective helper virus construct, pSEM, described above in Example 2, contains env sequences that limits its infectivity to rodent cells. It is, of course, desirable to be able to infect other types of cells, particularly human cells, with the recombinant virions, and in accordance with an important aspect of the present invention, amphotropic defective helper viruses are provided that are generally infective into mammalian cells, and particularly, that are infective into human cells. Construction of the DNA construct, pSAM, of such a helper virus from pSEM is diagrammatically illustrated in FIGURE 3. The construct utilizes segments of amphotropic murine retroviruses that are excised from 4070A clone (Chattopadhygy et al., J. Virol. 39, 779-791 (1982) and that have been shown to

infect not only rodent cell but cells of a large array of other species, including human cells. The most significant difference between pSAM and pSEM is the modification of pSEM to contain the env gene from the molecularly cloned amphotropic viral DNA. Thus, viruses that are rescued upon cotransfection with pSAM are able to infect both rodent and nonrodent cells, including human cells. Titers as high as 10^7 hHPRT units per ml. of virus rescued with pSAM vector have been obtained.

EXAMPLE 4

Mouse bone marrow cells were co-cultivated with rat cells A.D. Miller, et al., Proc. Natl. Acad. Sci. USA 80, 4709 (1983), producing HPRT-virus and helper virus (Moloney strain; MoMLV (H. Fan, J. Virol. 14, 421 (1974))), and then transplanted into syngeneic mice. Bone marrow cells were selected for infection because 1) it is likely that pluripotent self-renewing hematopoetic stem cells present in bone marrow are among those cells which can be infected R.J. Eckner, et al., J. Exp. Med. 149, 340 (1979), ii) it has been demonstrated that isologous bone marrow chimeras are highly sensitive to chronic MoMLV R.J. Eckner, et al., Virol. 122, 171 (1982), and iii) bone marrow cells are readily accessible and have a high proliferation rate after reintroduction into mice. To maximize the possibility of infection, the bone marrow cells were overlayed onto monolayers of virus-producing adherent cells for two days. The infected cells were then injected into lethally irradiated mice of the same strain as the donor mouse. The dose of radiation was chosen so that transplantation of fresh bone marrow was a requirement for survival of the mice. Thus certain hematopoetic cellular compartments (bone marrow and spleen) depleted by irradiation are repopulated by the virus-infected bone marrow cells.

Bone marrow cells from the spine of a C57BL/6 mouse were removed R.J. Eckner, et al., Virol. 122, 171

(1982), and aliquots of 5×10^7 nucleated cells were overlayed into 10 cm dishes of confluent 208F rat cells that produce $HPRT^+$-virus and MoMLV helper virus A.D. Miller, et al., Proc. Natl. Acad. Sci. USA 80, 4709 (1983). The rat cells produce virus such that medium harvested from these cells after 16 hr contains about 5×10^3 $HPRT^+$ cfu/ml with a ten-fold excess of helper virus. Viral infections were performed in Dulbecco-Vogt modified Eagle's medium containing 8 µg/ml Polybrene (Sigma Chemical Co.) and 10^{-5} M 2-mercaptoethanol. After 16 h, non-adherent cells were removed, resuspended at 2×10^7 viable cells/ml (85% of the cells were viable as judged by trypan blue dye exclusion), and 0.5 ml aliquots were injected intravenously into lethally irradiated (1200 R from a ^{60}Co source) C57BL/6 recipient mice. Irradiated control mice not receiving bone marrow all died of hematopoetic failure within 15 days after irradiation.

Two months after the bone marrow transplant, protein from spleen was analyzed electrophoretically for the presence of human HPRT. In the spleen from the surviving mouse receiving infected bone marrow, a form of HPRT which comigrates with one human HPRT isoenzyme is clearly visible. On longer exposures, a band comigrating with the other human isoenzymes can also be detected.

These results indicate that the human HPRT gene can be expressed in transplanted mouse bone marrow cells following co-cultivation with HPRT-virus producing cells.

EXAMPLE 5

A technical problem with the experiment described in Example 4 was the low survival rate (10-20%) of the irradiated recipient mice. Poor survival appeared to be due to loss of viability of bone marrow stem cells able to rescue recipient mice from hematopoetic failure after long term co-cultivation, as bone marrow maintained for 3 hours in vitro and used for

transplantation resulted in nearly 100% survival. To infect a high proportion of cells in this relatively short time period, a higher titer virus stock than was available from the existing virus-producing line was required.

To generate a cell line producing a high titer of HPRT-virus, construct pLPL and the packaging vector pSAM were cotransfected, F.L. Graham, A.J. van der Eb, Virology 52, 456 (1973); M. Wigler, et al., Cell 14, 725 (1978); C.M Corsaro, M.L. Pearson, Somatic Cell Genetics 7, 603 (1981), into $HPRT^-$ Balb/3T3 cells, and $HPRT^+$ clones producing high titer virus capable of converting $HPRT^-$ mouse or human cells to $HPRT^+$ were selected. (Ouabain resistant $HPRT^-$ (6-thioguanine resistant) Balb/3T3 cells (B77/OTG) were a gift from Dr. Harvey Ozer and were transformed with Rous sarcoma virus and then selected for resistance to ouabain and 6-thioguanine.) Culture medium exposed to a confluent monolayer of cells derived from one clone (c7c1) for 12 h contains about 10^7 HPRT-virus per ml, when assayed on $HPRT^-$ rat cells, $HPRT^-$ human (LNSV cells) or $HPRT^-$ Balb/3T3 cells as indicator cells. Because the virus released from c7c1 cells is capable of infecting human or mouse cells, it has the properties of an amphotropic virus. Naturally occurring amphotropic viruses, including the virus generated from the molecular clone used in the construction of pSAM, are N-tropic, i.e., have reduced ability to infect cells of Balb/c mouse origin. However, the virus produced by c7c1 can infect Balb/3T3 cells with high efficiency, as expected N. Teich, in RNA Tumor Viruses, R. Weiss, et al., eds. (Cold Spring Harbor Laboratory, New York) Ch. 2, pp209-260 (1982), due to the presence of the NB-tropic p30gag region from Mo-MLV in pSAM.

The HPRT-virus produced by c7c1 had high titer and a wide host range. Virus produced by c7c1 contains less than 0.1% of helper virus, an insignificant titer.

The high titer amphotropic HPRT-virus obtained from clone c7c1 was used to infect mouse bone marrow cells. Mouse bone marrow cells were infected using HPRT-virus harvested from a clonal Balb/3T3 cell line (c7c1) cotransfected with the HPRT expression vector pLPL and the packaging vector pSAM. The titer of the virus stock used was 2 x 10^7 HPRT-virus/ml. Bone marrow cells obtained from mouse spine were exposed to HPRT-virus in two one-hour incubations at room temperature. 100 ml virus stock was mixed with 5 x 10^8 bone marrow cells. After each incubation, the cells were centrifuged at 600 g for 10 min and the medium removed. Prior to injection, the cells were suspended in 10 ml medium containing 10^7 MoMLV helper virus. 0.5 ml doses of suspended bone marrow cells were injected into the tail veins of mice previously exposed to 1200 R from ^{60}Co radiation source.

After 3 months, spleen and bone marrow (spine) cells were collected and overlayed on recipient cells for virus assay. HPRT-virus assay was performed by overlay of 10^7 mouse cells on 5 x 10^5 $HPRT^-$ ouabain resistance (oua^r) Balb/3T3 cells in a 5 cm dish. After 16 h, the dishes were washed 3 times to remove loosely adherent mouse cells, trypsinized, and split 1:5 into medium containing 30 μM hypoxathine, 1 μM amethopterin, 20 μM thymidine (HAT medium) and 5 mM ouabain.

Colonies were counted after 6-10 days. MoMLV helper virus was quantitated using the XC plaque assay, W.P. Rowe, W.E. Pugh, J.W. Hartley, Virology 42, 1136 (1970). Briefly, 10^7 mouse cells were overlayed on 5 x 10^5 NIH 3T3 TK^- cells for 16 h in a 5 cm dish. The dishes were washed 3 times and split 1:10. Three days later the cells were UV irradiated and overlayed with XC cells. Two days later, the cells were stained and placques were counted. The amphototropic virus is XC^- and does not score in this assay. Amphotropic

virus was quantitated using the S^+L^- assay, R.J. Eckner, K.L. Hettrick, J. Virol. 24, 383 (1977). 10^7 mouse cells were overlayed on 10^5 CCC-81 cat cells in a 5 cm dish. After 16 h, the dish was washed and the cells were split 1:10 into dishes containing 2×10^5 rat kidney (NRK) cells. After 5 days, transformed foci resulting from the helper virus-mediated transfer of the sarcoma virus present in the cat cells into the NRK indicator cells were counted. MoMLV does not score in this assay because of its inability to infect cat cells, and thus rescue the replication defective transforming virus harbored by these cells. Titers of MoMLV were approximately 10^6 XC plaque forming units per 10^7 cells per 16 h and the amphotropic virus ranged from $10^3 - 7 \times 10^3$ focus forming units per 10^7 cells per 16 h. This shows that a chronic infection is induced in the recipient mice.

The survival rate of these mice (no deaths in 20 mice receiving transplants) was much higher than than of the mice receiving bone marrow after 12-24 h cocultivation with virus producing cells. Irradiated mice that did not receive bone marrow transplants died within 10 days. Spleen and bone marrow cells from some of the mice receiving infected bone marrow were analyzed for production of HPRT-virus. One expects that some hematopoietic cells from the mice receiving infected bone marrow should be infected with both the HPRT-virus and helper virus, and thus should be producing HPRT-virus. Spleen and bone marrow cells from mice receiving bone marrow transplants were removed and overlayed onto adherent $HPRT^-$ ouabain resistant (oua^r) cultured mouse cells. One day later, the dishes were washed thoroughly to remove unattached cells, and the cells were exposed to selective medium containing HAT and ouabain. This selection ensures that only cultured cells converted to $HPRT^+$ by virus will survive, as ouabain kills the donor mouse cells, and HAT

selects against HPRT$^-$ cells. The development of colonies of cells surviving HAT selection are thus dependent on HPRT-virus production by the donor cells. HPRT-virus continued to be produced by spleen and bone marrow cells of mice infected with the high titer HPRT-virus even after 133 days following transplantation, and demonstrates chronic persistence of the HPRT-virus in these mice.

DNA from cultured cells converted to HPRT$^+$ by exposure to spleen or bone marrow from mice receiving bone marrow transplants was analyzed for the presence of HPRT-viral DNA to determine if the HPRT-virus was indeed responsible for colony formation and that no rearrangement of the virus had occurred. Cultured cells converted to HPRT$^+$ by the same virus stock used to infect mouse bone marrow was also analyzed. Cellular DNA was digested with SstI cuts in both LTRs of the virus; thus a single band of about 3.9 kb was expected to be present in all infected cells, and the HPRT-specific radiolabeled probe hybridized to a DNA species of about 3.9 kb from cells infected with stock virus from c7c1 cells. However, there was no hybridization to a similar size DNA in uninfected cells. An additional band of about 9 kb representing the endogenous mouse HPRT DNA could also be seen in both samples. The 3.9 kb band representing the HPRT-viral DNA is present in cells converted to HPRT$^+$ by exposure to cells from infected mice. Thus viral DNA is present in colonies of HPRT$^+$ cells produced by exposure to cells from infected mice, and the virus has the same organization and is apparently identical to that originally used for infection of the mice.

Results presented here show that retroviral vectors can be used to transfer genetic material into intact animals. Two types of observations justify this claim: i) bone marrow and spleen cells of mice receiving infected bone marrow became chronic producers

of HPRT-virus, receiving infected bone marrow became chronic producers of HPRT-virus, additionally, the released virus appeared to be identical to the virus used for infection of the engrafted bone marrow, and ii) human HPRT was expressed in the spleen of a recipient mouse.

This experiment further demonstrates that gene therapy can be performed by using a recombinant retrovirion to carry a gene into a transplantable cell line and then transplanting the cell line into a living animal.

Lesch-Nyhan disease is a devastating human disease which is apparently caused by a defect in the HPRT gene W.N. Kelley, J.B. Wyngaarden, in The Metabolic Basis of Inherited Disease, 5th edition (ed. J.B. Stanbury et al.) McGraw Hill (1983); C.T. Caskey, G.D. Kruh, Cell 16, 1 (1979). One of the long term goals of HPRT-research is to test the utility of retroviral vectors as potential agents for correction of this defect in somatic cells. It has recently shown that lymphoblasts from Lesch-Nyhan patients can be infected with the HPRT-virus in vitro R.C. Willis, et al., J. Biol. Chem., 259, 7892 (1984). The utility of retroviral vectors for somatic cell modification may require the availability of cell lines which provide high titers of retrovirus containing foreign genes which are capable of infection but not further propagation. The amphotropic packaging construct described here only partially meets these criteria. However, there has not been observed any disease or gross pathology in infected mice. In addition, the HPRT-virus was designed so that no viral proteins are synthesized, thus minimizing the possibility of antigenic rejection of HPRT-virus-infected cells.

Although in this experiment, the gene carrying cells were introduced in replacement of substantially destroyed bone marrow tissue, techniques are available

for enhancing the relative proportion of genetically altered transplanted somatic cells relative to the endogenous population of cells. For example, a retroviral vector may carry, in addition to a gene of interest, a dihydrofolate reductase (dhfr) gene. If bone marrow cells infected with the retrovirion are introduced into the animal and then the animal is administered controlled doses of methotrexate, the transplanted, gene-carrying bone marrow cells should exhibit a selective advantage relative to the endogenous bone marrow cells.

EXAMPLE 6

In this example, the construct pLPL, described above in Example 1, was further modified to incorporate a rat growth hormone "mini-gene" under its own promoter. Construction of the mini-gene is illustrated in FIGURE 4A, and its insertion into the pLPL construct is illustrated in FIGURE 5A. Briefly, the mini-gene (FIG. 4B) consists of rat growth hormone cDNA produced from rat growth hormone messenger RNA by reverse transcriptase. To the cDNA is linked the normal rat growth hormone promoter sequence. The cDNA contains no intervening sequences (introns) which might prevent the gene from being correctly transcribed in foreign cells into which it is introduced. The pLPL construct is cut at the Hpa I restriction site, and at this site, the rGH mini-gene obtained from a growth mini-gene carrying plasmic (pGH) by cleavage with Hind III is inserted by blunt end ligation after filling in the ends using the Klenow fragment. By this procedure, it is expected that some of the mini-gene will be inserted in parallel with the viral LTR promoter sequences and that some of the mini-gene will be inserted in reverse orientation with respect to the viral LTR promoter sequences. The viral genome corresponding to parallel insertion is shown in FIGURE 5b.

DNA constructs representing each orientation of

the minigene, LPGHL (parallel), and LPHGL reverse, were introduced (10 μg/5 cm dish of cells) into 208F $HPRT^-$ rat cells by calcium phosphate precipitation (exp. 1). One day after transfection, the cells were trypsinized and seeded into HAT medium. $HPRT^+$ colonies were visible in about 5 days, and were pooled for analysis. For assay of GH production, the cells were seeded at about 20% confluency in growth medium containing 10% fetal bovine serum with no added inducers (control) or with 10^{-6} M dexamethasone (dex) or 10^{-6} M dex and 10^{-7} T_3. After 3 days, GH in the medium was measured using an NAIMDD radioimmunoassay kit and rat GH standard NIAMDD-rGH-I-4, Doehmer, J. et al., Proc. Natn. Acad. Sci USA, 79: 2268-2272 (1982). Results are expressed in Table 2 in ng GH per ml of medium.

Fold induction was calculated as the ratio of GH production with dex + T_3 compared to control (GH production from uninduced cells). Fetal bovine serum contains low levels of inducers of GH production, thus the fold induction may be underestimated.

In a separate experiment (exp. 2), the transfected cells were infected with MoMLV helper virus (MLV-K, ref. 31) passaged twice, and virus production was measured in medium harvested under 16 h from a confluent dish of cells. $HPRT^+$ colony forming units per ml of medium (cfu/ml) were measured on 208F rat cells and MoMLV plaque forming units per ml (pfu/ml) on NIH 3T3 TK^- cells.

TABLE 2

ANALYSIS OF TRANSFECTED CELLS FOR VIRUS RESCUE AND GROWTH HORMONE PRODUCTION

Transfected DNA	Growth Hormone Production				Virus Rescue	
	Control	dex	dex+T_3	Fold-Induction	$HPRT^+$ cfu/ml	MoMLV pfu/ml
pLPGHL						
Exp. 1	11	23	24	2.2	6.5×10^2	8×10^4
Exp. 2	22	102	99	4.5	3.2×10^2	8×10^4
pLPGHL						
Exp. 1	29	83	101	3.5	2.4×10^2	7×10^4
Exp. 2	35	70	75	2.1	2.3×10^2	8×10^4
no DNA		<2	<2	<2	-	-

Table 2 shows that these pooled transfectants secrete significant levels of growth hormone. Additionally, these transfected cells synthesize higher levels of growth hormone following induction with dexamethasone (dex) or with dex plus T_3 (Table 2). Thus the HPRT-GH-virus construct is capable of directing the synthesis of GH after transfection into cells.

The HPRT-GH-virus construct is incapable of synthesizing viral proteins, so helper virus is required to rescue this replication defective virus. It was attempted to rescue virus containing the HPRT and GH genes by superinfecting the transfected cells with Moloney murine leukemia virus (MoMLV). Both the HPRT-GH-virus and the MoMLV helper virus should be packaged into virions by proteins synthesized by the helper virus. Table 2 shows that infectious virus transmitting the $HPRT^+$ phenotype is indeed rescued from the transfected, MoMLV superinfected cells. The ratio of HPRT-GH-virus to helper virus is similar to that previously reported for the parent HPRT-virus, Miller et al., Proc. Natn. Acad. Sci. USA 80, 4709-4713 (1983), indicating that the presence of the GH minigene does not affect virus rescue.

To determine whether the GH minigene was being cotransferred in the rescued virus along with HPRT,

virus rescued from cells transfected with pLPGHL or pLPHGL was used to infect 208F rat cells and BALB 3T3 mouse cells. 16 independent $HPRT^+$ clones were isolated and characterized for GH production. Because GH is secreted from cells, it provides a convenient and sensitive means to monitor the expression of the transferred gene. Table 3 shows that of the 8 $HPRT^+$ rat clones and 8 $HPRT^+$ mouse clones, at least half synthesize significant amounts of GH. In the remaining infectants, either the minigene is inactive or failed to co-transfer with the HPRT gene.

TABLE 3

GH PRODUCTION AND INDUCTION IN CELLS INFECTED WITH VIRUS RESCUED FROM TRANSFECTED CELLS

Rescued virus	HPRT+ clone number	Growth hormone production* Control	dex	dex+T_3	Fold-Induction
A. 208F Rat Cell Infectants					
LPGHL	F0.2	6.0	22	30	5.0
	F0.3	6.5	7.9	8.9	1.4
	F1.2	13	20	20	1.5
	F1.3	3.1	2.4	2.7	(0.9)
LPHGL	F2.1	3.3	3.6	2.9	(0.9)
	F2.4	4.0	3.7	2.4	(0.6)
	F3.2	25	93	126	5.0
	F3.3	4.5	4.4	4.0	(0.9)
uninfected cells		<1.5	<1.5	<1.5	-
B. Balb/3T3 Mouse Cell Infectants					
LPGHL	B0.2	12	10	12	1.0
	B0.4	12	10	16	1.3
	B1.2	3.5	7.60	8.6	(2.5
	B1.4	2.7	5.9	6.8	(2.5)
LPHGL	B2.3	<1	<1	<1	-
	B2.4	<1	<1	<1	-
	B3.3	236	246	289	1.2
	B3.4	<1	<1	<1	-
uninfected cells		<1	<1	<1	-

*GH production from induced or uninduced cells was measured as described in Table 1. HPRT+ colonies induced by virus rescued from transfected 208F cells (Table 1) were isolated using cloning cylinders. The first letter of the clone designation refers to the cell type of the infectant (F for 208F rat cells or B for Balb/3T3 mouse cells). Fold-induction values which may not be significant, due to limitations of the assay at low GH levels, are indicated in parentheses.

The endogenous GH gene in cultured pituitary cells and in the animals has been shown to be transcriptionally regulated by both glucocorticoid and thyroid hormones, although the potential sequences

involved in this regulation have not been localized Evans et al., Proc. Natn. Acad. Sci. USA 79: 7659-7663 (1982), Spindler et al., J. Biol. Chem., 257: 11627-11632 (1982). Two of the rat infectants (clones FO.2 and F3.2 Table 3A) are clearly responsive to dex, and this response is potentiated by T_3. Expression and regulation appears to be independent of the transcriptional orientation of the GH minigene. In addition, two of the mouse clones appear to respond to dex and T_3 (clones B1.2 and B1.4, Table 3B), although the levels of GH production are low.

Southern blot analysis provides an alternative approach to follow the propagation of infectious virus containing both HPRT and the GH minigene that is not dependent upon expression. Therefore the DNA structure of the integrated viruses was examined in several of the infectants. Retroviruses integrate into cellular DNA in a linear fashion and are flanked by LTRs. Thus, the GH minigene should always be flanked by viral LTRs although the integration site in the cellular genome is likely to be random. DNA extracted from several viral infectants was cleaved with the restriction enzyme which cuts only once in the HPRT-GH-provirus. The resulting fragments were analyzed by the technique of Southern, Southern E. M., J. Mol. Biol. 98, 503-517 (1975), using a radio-labelled GH probe. The GH probe hybridizes to a single band in uninfected mouse and rat cells, presumably derived from the endogenous GH gene, while in DNA prepared from five infectant clones an additional band was detected. The DNA fragment containing the HPRT-GH-provirus has a different size in each of the infectants, as expected, due to the random occurrence of BamHI sites in the cellular DNA near the virus integration site. The results also show that each infectant contains a single integrated provirus, as only one new band is present in DNA from each of the clones. Similar results were obtained using HPRT sequences as a probe.

Finally, DNA from these clones was digested with SstI and subjected to the same analysis. SstI cuts once in each LTR of the virus and should reveal the size of the integrated HPRT-GH-provirus. In DNA from each of the infectants, there is a single new band in addition to the endogenous band. As expected, the new bands have the same size and all match the expected size (5.3 kb) of an integrated HPRT-GH-provirus. Thus, no major rearrangement of the viral genome has occurred during generation of virus or of infection of these clones. It is concluded that the simultaneous propagation of HPRT and the GH minigene is independent of transcriptional orientation of the later relative to the viral LTR.

To examine transcription from the HPRT-GH-provirus, combinations of RNA blot and S1 nuclease analysis were performed. Northern blot analysis of growth hormone mRNA synthesized by the integrated virus in several infected cell lines was performed. The major mRNA species produced in all of the infectants is the same size (1kb) regardless of the transcriptional orientation of the minigene. Furthermore, this transcript is identical to the RNA product of the endogenous GH gene in rat pituitary cells. S1 nuclease analysis was used to characterize the 5' terminus to determine whether the appropriate GH transcriptional signals were being utilized. The results show that mRNA produced from the endogenous GH gene protects a 189 nt fragment spanning the promoter region and identifies a 5' cap site 25 nt from the sequence TATAA. An identical size fragment is protected by mRNA obtained from HPRT-GH-virus infected mouse or rat cells, thus confirming accurate initiation from the minigene. Furthermore, S1 nuclease and mRNA size analysis imply that the GH polyadenylation signals are being used.

RNA analysis confirms that the GH minigene, in addition to containing functional initiation and

polyadenylation sites, also remains inducible with dex and T3. mRNA induction parallels increases in GH production (Table 3). For example, rat clone F3.2 (Table 3A) exhibits a 5-fold induction of both GH mRNA and secreted protein following induction. Clone F0.2 exhibits similar changes in both RNA and GH synthesis. On the other hand, mouse clone B3.3 (Table 3B) synthesizes constituitive and relatively high levels of GH, which remains unaffected by hormone administration. Correspondingly high GH mRNA levels also remain unaltered. Some clones, such as B1.4 and F1.2, reveal low levels of both GH RNA and secreted protein.

Although in this example the mini-gene was assembled from a cDNA that encodes the rGH protein and from the rGH promoter sequence, it is to be understood that the gene-encoding sequence may be linked to and under the translational control of a promoter sequence to which it is not linked in nature. A gene-encoding sequence, for example, might be linked to a promoter sequence derived from another species, particularly where a protein endogenous to one species is to be raised in the cells of another species.

The invention, as exemplified by the above examples, substantially expands the utility of retroviruses as vectors for carrying genetic information into foreign cells. Through the use of helper viruses encoding amphotropic packaging proteins, recombinant retrovirion are generated which are generally useful for carrying genetic information into vertebrate cells. Novel defective helper viruses are provided for generating recombinant retrovirions that are useful for a one-time infection and that are free of infectious helper virus. Unique constructs are provided incorporating proviral LTR sequences but deleting sequences, e.g., poly A sequences, which tend to limit transcription, thereby improving the utility of the retroviral vectors for carrying and expressing foreign

DNA sequences.

Further provided by the invention are cells that have been genetically altered by recombinant retroviral vectors and are shown to continue to express gene-product after the cells are transplanted into a living animal. Retroviral vectors, including helper-free vectors, are shown to genetically alter somatic cells of a living animal by the most simple techniques, such as directly infecting the animal intraveneously with a suspension of recombinant retroviral vectors. Both implantation of genetically altered cells and genetic alteration of somatic cells by direct infection are important gene therapy tools.

Foreign genes may be inserted into retroviral constructs either under the promotional control of the strong viral LTR promoters or under the control of linked non-viral promoter sequence. Both types of promotional control have advantages in particular situations.

While the invention has been described with reference to certain preferred embodiments, modifications obvious to one with ordinary skill in the art may be made without departing from the scope of the present invention.

Various features of the invention are set forth in the following claims.

1. In combination, A) a recombinant construct comprising a 5' retroviral LTR nucleotide sequence including a cell genome insertion site, a genome packaging sequence linked to said 5' LTR sequence, a promotor sequence, a heterologous protein-encoding sequence under promotional control of said promoter sequence and a 3' retroviral LTR sequence including a cell genome insertion site, said construct being deficient in viral protein-encoding sequences needed to package the corresponding RNA retroviral genome as a complete retrovirion; and B) a helper retroviral vector useful for rescuing the retroviral genome corresponding to said recombinant construct, said helper retroviral vector comprising sequences encoding the requisite packaging proteins for packaging said retroviral genome, said packaging protein-encoding sequences of said helper retroviral vector including a sequence that encodes an amphotropic envelope protein, and a promoter sequence or sequences linked to said packaging protein-encoding sequences for expression of packaging proteins, said helper retroviral vector being deficient in its own packaging sequences.

2. A combination in accordance with claim 1 wherein said recombinant construct encodes no viral packaging proteins and wherein said helper retroviral vector encodes the full complement of proteins necessary for packaging said retroviral genome.

3. A helper retroviral vector being useful for rescuing a retroviral genome corresponding to a recombinant DNA construct that is deficient in packaging protein-encoding sequences, said helper retroviral vector comprising nucleotide sequences encoding the requisite packaging proteins that are lacking from the construct for packaging the retroviral genome, said packaging protein-encoding sequences of said helper

retroviral vector including a sequence that encodes an amphotropic envelope protein and a promoter sequence or sequences linked to said packaging protein-encoding sequences for expression of packaging proteins, said helper retroviral vector being deficient in its own packaging sequences.

4. A helper retroviral vector in accordance with Claim 3 useful for rescuing a retroviral genome corresponding to a recombinant DNA construct that encodes no packaging proteins, said retroviral vector having sequences that encode the full complement of proteins necessary for packaging said genome.

5. A titer of infectious retrovirions, which retrovirions each comprise a recombinant genome having a 5' LTR sequence including a cell genome insertion site, a promoter sequence, a heterologous protein-encoding sequence under the promotion control of said promoter sequence and a 3' retroviral LTR sequence including a cell genome insertion site, said genome being deficient in viral protein-encoding sequences needed to package progeny genomes as complete retrovirions, and a full complement of packaging proteins packaging said genome for said retrovirion to be infective of eukaryotic cells, said packaging proteins of each retrovirion including an amphotropic envelope protein, whereby said recombinant retrovirions are infective across biological families, said packaging proteins being provided by a helper retroviral vector to the extent that said genome is deficient in viral packaging protein-encoding sequences, said titer being free of helper retroviral vector.

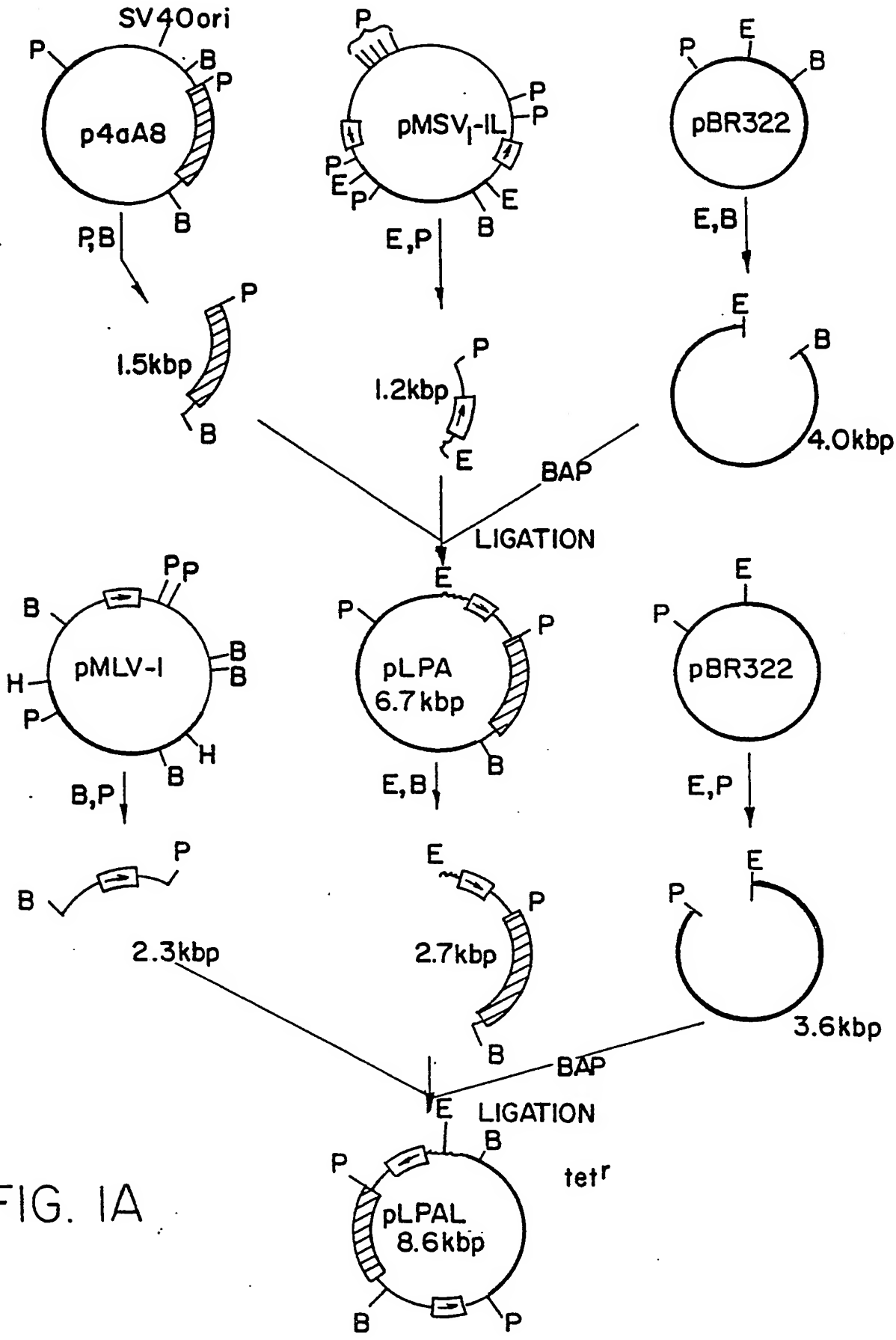
6. A titer according to Claim 5 wherein said genome encodes no retroviral packaging proteins and all of said packaging proteins are provided by the helper retroviral vector.

7. A titer according to Claim 6 wherein said retrovirions have amphotropic envelope proteins which make said retrovirons infective of human cells.

8. A method of imparting a genetic characteristic to a selected eukaryotic cell through a retrovirion infection comprising assembling a recombinant DNA construct comprising a 5' viral LTR sequence including a cell genome insertion site, a packaging sequence, a promotor sequence, a heterologous protein-encoding sequence under the promotional control of said promoter sequence and a 3' viral LTR sequence including a cell genome insertion site, said construct being deficient in viral protein-encoding sequences needed to package the corresponding retroviral genome as a complete retrovirion, including being deficient an envelope protein-encoding sequence, providing a helper retroviral vector having nucleotide sequences encoding the viral packaging proteins, including an amphotropic envelope protein, needed to package said retroviral genome and sequences promoting expression of said viral packaging protein-encoding sequences, said helper retroviral vector being deficient in its own packaging sequence, introducing said construct into a host eukaryotic cell, rescuing the genome corresponding to a said construct as an infectious retrovirion from said host cell with said helper retroviral vector, and infecting said selected eukaryotic cell with said retrovirion.

9. A method according to claim 8 wherein said host eukaryotic cell and said selected eukaryotic cell are from species of different biological families.

10. A method according to Claim 9 wherein said host eukaryotic cell is a murine cell and said selected eukaryotic cell is a human cell.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P
B
P
B
P,B
1.5kbp
P
B
pMSV$_I$-IL
P
P
P
E
P
E
B
E,P
1.2kbp
P
E
pBR322
E
P
B
E,B
E
B
4.0kbp
BAP
LIGATION
pMLV-I
P
P
B
B
H
B
P
B
H
B,P
2.3kbp
P
B
pLPA
6.7kbp
E
P
P
B
E,B
2.7kbp
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P
B
pBR322
E
P
E,P
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LIGATION
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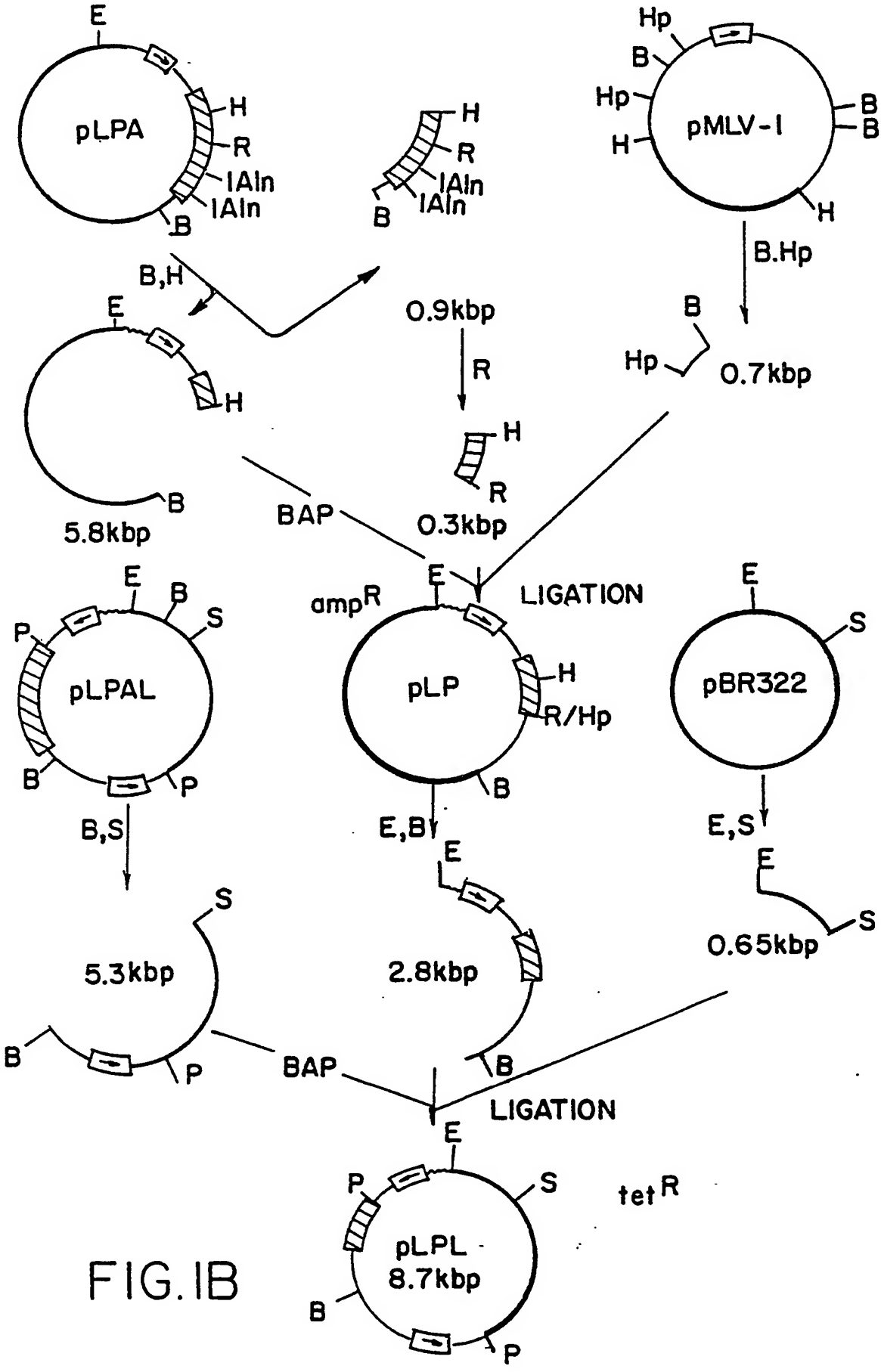
B
P
B
P
tet^r

FIG. IA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E
H
R
IAIn
IAIn
B
pMLV-1
Hp
B
Hp
H
B
B
H
B.Hp
B,H
E
H
B
5.8kbp
0.9kbp
R
H
R
0.3kbp
B
Hp
0.7kbp
BAP
LIGATION
ampR
E
pLP
H
R/Hp
B
E,B
pLPAL
E
B
S
P
B
P
B,S
5.3kbp
S
B
P
pBR322
E
S
E,S
E
S
0.65kbp
2.8kbp
E
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B
P
tetR

FIG. 1B

FIG.2.

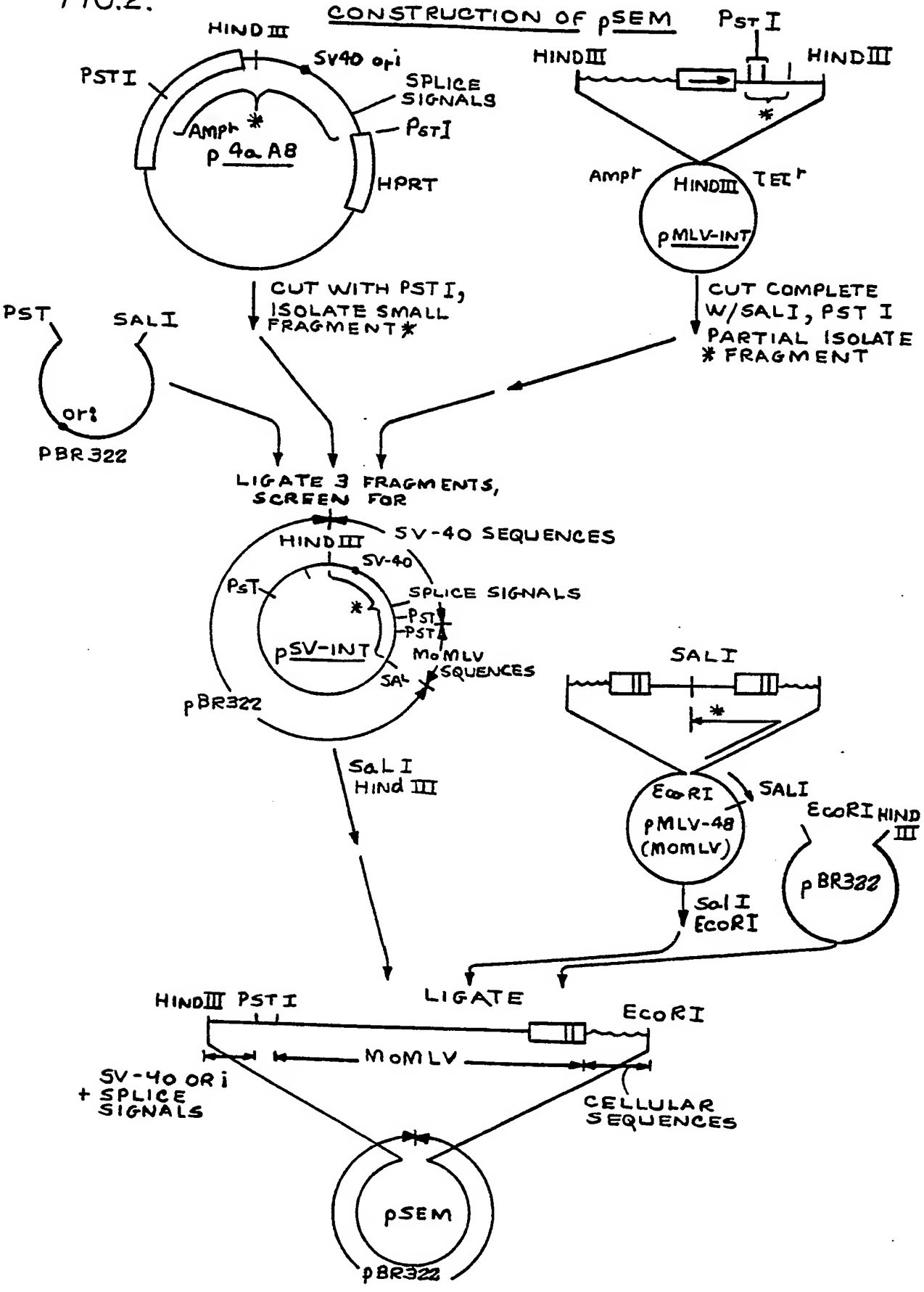

CONSTRUCTION OF pSEM
HIND III
PST I
SV40 ori
SPLICE SIGNALS
Amp r
p 4a A8
HPRT
HIND III
HIND III
Amp r
HIND III
Tet r
pMLV-INT
CUT WITH PST I, ISOLATE SMALL FRAGMENT *
CUT COMPLETE W/SAL I, PST I PARTIAL ISOLATE * FRAGMENT
PST
SAL I
ori
PBR322
LIGATE 3 FRAGMENTS, SCREEN FOR SV-40 SEQUENCES
HIND III
SV-40
SPLICE SIGNALS
pSV-INT
MoMLV SEQUENCES
pBR322
SAL I
SAL I
HIND III
EcoRI
SAL I
pMLV-48 (MoMLV)
EcoRI HIND III
pBR322
Sal I
EcoRI
LIGATE
HIND III PST I
EcoRI
MoMLV
SV-40 ORi + SPLICE SIGNALS
CELLULAR SEQUENCES
pSEM
pBR322

FIG. 3.

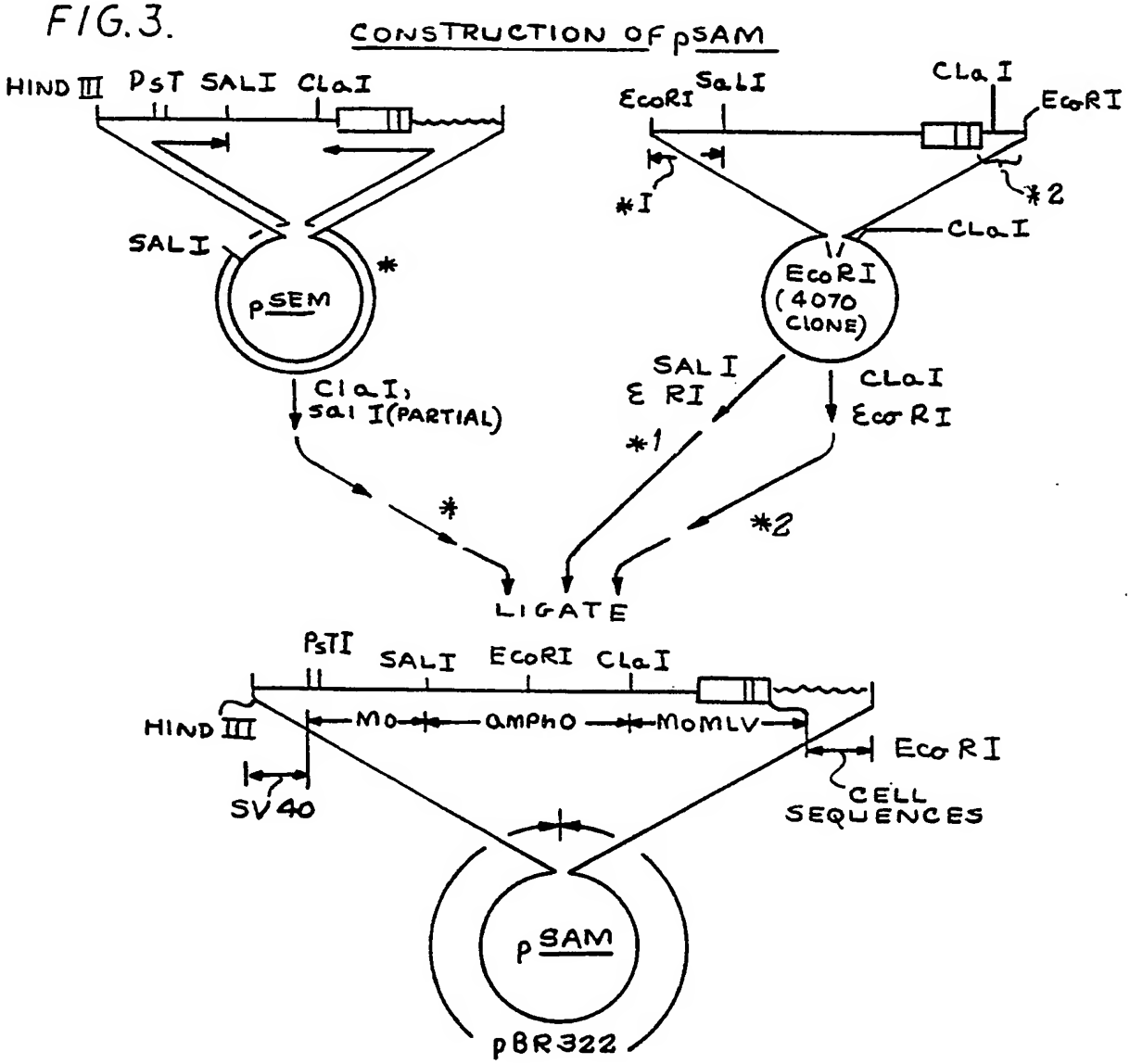
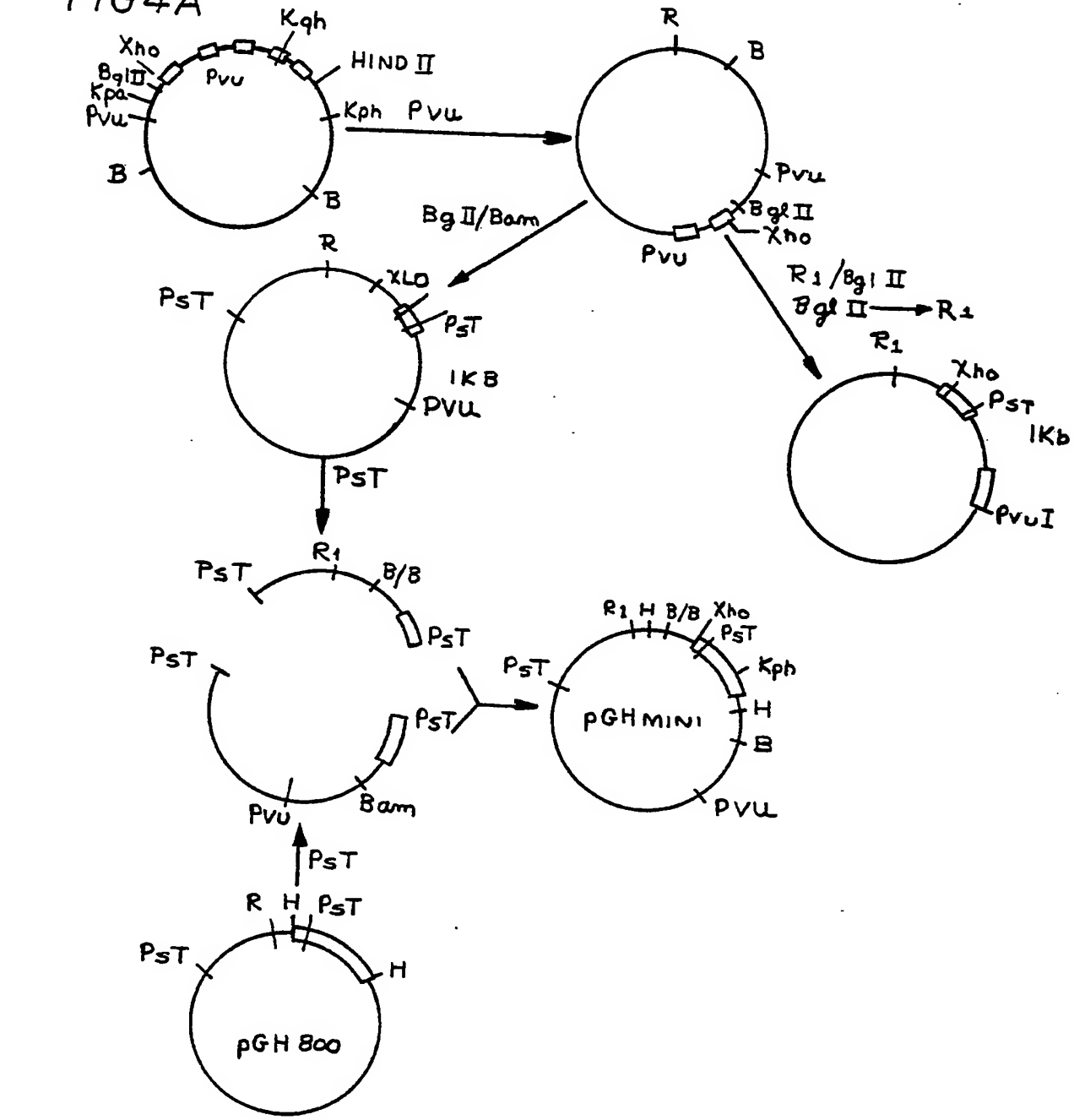

CONSTRUCTION OF pSAM
HIND III PsT SALI ClaI
SALI
pSEM
ClaI, sal I(PARTIAL)
EcoRI SalI
ClaI
EcoRI
*I
*2
ClaI
EcoRI (4070 CLONE)
SAL I E RI
ClaI EcoRI
*1
*2
LIGATE
PsTI SALI EcoRI ClaI
HIND III
MO AMPHO MoMLV
EcoRI
SV40
CELL SEQUENCES
pSAM
pBR322

FIG4A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Xho
BglII
Kpa
Pvu
HIND II
Pvu
Kph Pvu
B
B
R
B
Pvu
BglII
Xho
Pvu
Bg II/Bam
R1/Bgl II
Bgl II → R1
R
XLO
PsT
PsT
IKB
PVU
PsT
R1
Xho
PsT
IKb
PvuI
PsT
R1
B/B
PsT
PsT
PsT
Bam
PVU
R1 H B/B Xho
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PsT
R H PsT
PsT
H
pGH 800

FIG.4B.

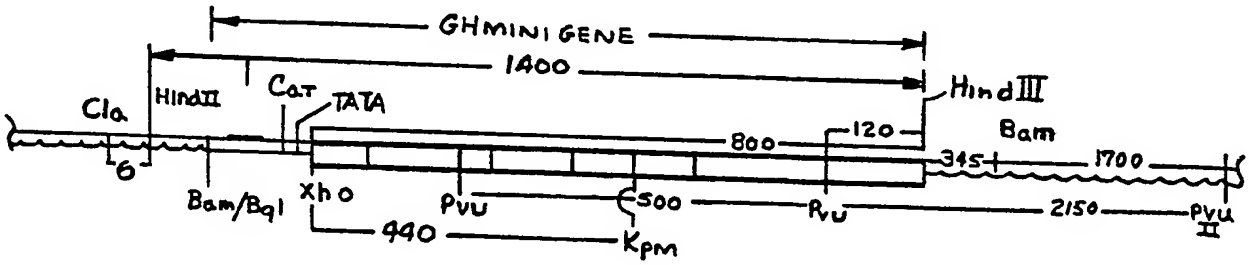

GHMINI GENE
1400
HindII
CAT
TATA
Cla
Hind III
800
120
Bam
345
1700
6
Bam/Bgl
Xho
PVU
500
RV
2150
PVU II
440
Kpm

FIG.5A.

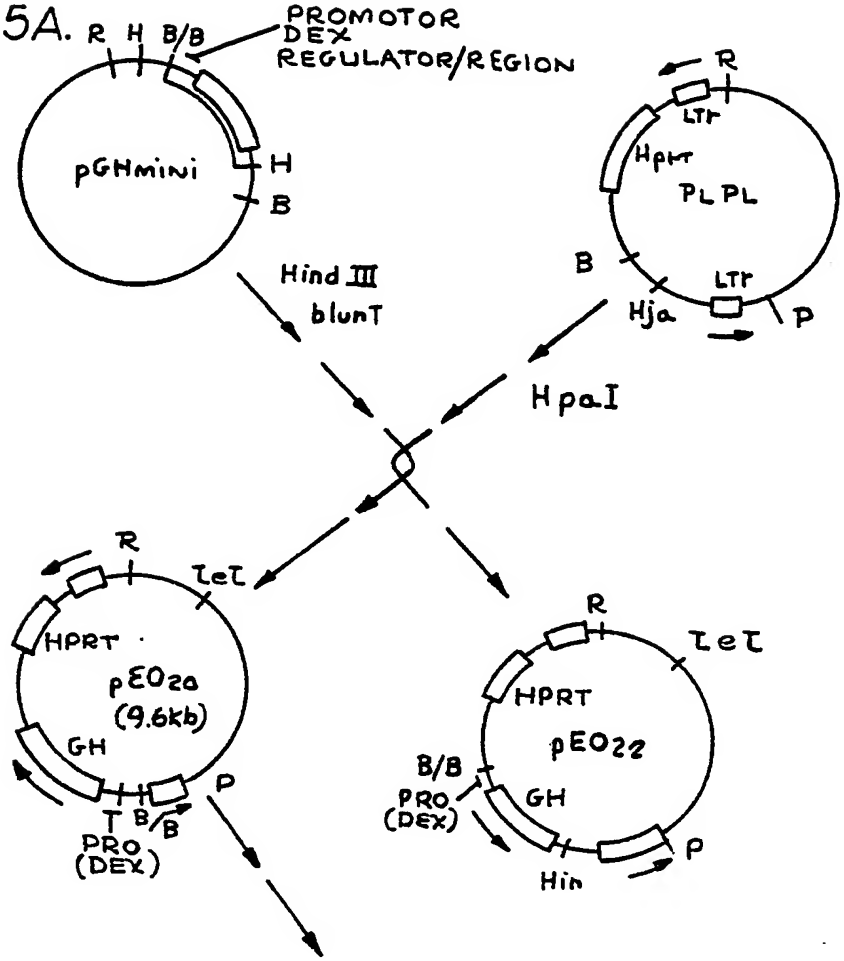
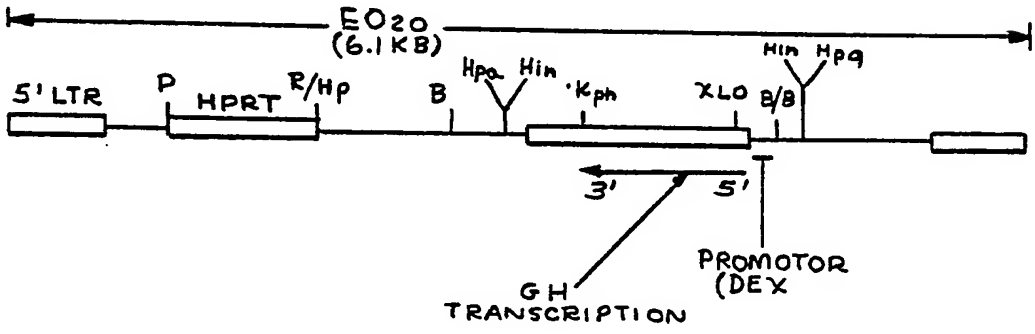

R H B/B
PROMOTOR
DEX
REGULATOR/REGION
pGHmini
H
B
Hind III
blunT
R
LTr
HpHT
PL PL
B
LTr
Hja
P
HpaI
R
tet
HPRT
pEO20
(9.6kb)
GH
T
B/B
PRO
(DEX)
P
R
tet
HPRT
pEO22
B/B
PRO
(DEX)
GH
P
Hin

FIG.5B.


EO20
(6.1 KB)
5' LTR
P
HPRT
R/Hp
B
Hpa
Hin
Kph
XLO
B/B
Hin
Hpa
3'
5'
GH
TRANSCRIPTION
PROMOTOR
(DEX

INTERNATIONAL SEARCH REPORT

International Application No PCT/US85/01442

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [3]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC: 4 C12N 1/00, C12N 7/00, C12N 7/04, C12N 15/00

II. FIELDS SEARCHED

Minimum Documentation Searched [4]

Classification System	Classification Symbols
U.S.	435/68,70,71,91,172.3,235,236,239,317; 536/27; 424/89

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [5]

CA SEARCH DATABASE: 1977-85

III. DOCUMENTS CONSIDERED TO BE RELEVANT [14]

Category *	Citation of Document, [16] with indication, where appropriate, of the relevant passages [17]	Relevant to Claim No. [18]
Y	US,A, 4,405,712 Published 20 September 1983, Vande Woude et al.	1-10
X	N,Willis et al, J.Biol.Chem., Vol. 259, June 1984, pages 7842-7849	1-10
Y	N,Brennand et al, J.Biol.Chem., Vol.258, August 1983, pages 9593-9596.	1-10
Y	N,Tabin et al, Molecular and Cellular Biology, April 1982, pages 426-436	1-10

* Special categories of cited documents: [15]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search [2]	Date of Mailing of this International Search Report [2]
02 October 1985	10 OCT 1985
International Searching Authority [1]	**Signature of Authorized Officer [20]**
ISA/US	James Martinell

Form PCT/ISA/210 (second sheet) (October 1981)

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

Y	N,Shimotohno et al, Cell, Vol 26, 1981, pages 67-76	1-10
Y	N,Wei et al, J.Virol., Vol. 39, September 1981, pages 935-944	1-10
Y,P	N,Anderson, Science, Vol. 226, October 1984, pages 401-409	1-10

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE [10]

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers....., because they relate to subject matter [12] not required to be searched by this Authority, namely:

2. ☐ Claim numbers....., because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international sparch can be carried out [13], specifically:

VI. ☐ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING [11]

This International Searching Authority found multiple inventions in this international application as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

☐ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional search fees.

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, [16] with indication, where appropriate, of the relevant passages [17]	Relevant to Claim No [18]
X	N,Miller et al, Proc. Natl. Acad. Sci. USA, Vol.80, August 1983, pages 4709-4713	1-10